Interactions of GGA3 with the ubiquitin sorting machinery

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The Golgi-localized, γ-ear-containing, Arf-binding (GGA) proteins constitute a family of clathrin adaptors that are mainly associated with the *trans*-Golgi network (TGN)[1–3] and mediate the sorting of mannose 6-phosphate receptors[4–6]. This sorting is dependent on the interaction of the VHS domain of the GGAs with acidic-cluster-dileucine signals in the cytosolic tails of the receptors[4,5]. Here we demonstrate the existence of another population of GGAs that are associated with early endosomes. RNA interference (RNAi) of GGA3 expression results in accumulation of the cation-independent mannose 6-phosphate receptor and internalized epidermal growth factor (EGF) within enlarged early endosomes. This perturbation impairs the degradation of internalized EGF, a process that is normally dependent on the sorting of ubiquitinated EGF receptors (EGFRs) to late endosomes. Protein interaction analyses show that the GGAs bind ubiquitin. The VHS and GAT domains of GGA3 are responsible for this binding, as well as for interactions with TSG101, a component of the ubiquitin-dependent sorting machinery. Thus, GGAs may have additional roles in sorting of ubiquitinated cargo.

The three mammalian GGAs (that is, GGA1, GGA2 and GGA3) have a modular organization, consisting of: first, a VHS (Vps27, HRS, and STAM) domain that recognizes acidic-cluster-dileucine signals present in the cytosolic tails of the mannose 6-phosphate receptors (MPRs)[4,5]; second, a GAT (GGA and TOM) domain that interacts with the GTP-bound form of ADP-ribosylation factors (Arfs)[1,2,7]; third, a hinge domain that binds clathrin[5,7]; fourth, a GAE (γ-adaptin ear) domain that interacts with accessory proteins[3,8–10]. These properties have led to the notion that the mammalian GGAs function as monomeric clathrin adaptors for the sorting of MPRs at the TGN. Consistent with this notion, GGA1 tagged with green fluorescent protein (GFP) localizes to the TGN and clathrin-containing carriers that move from the TGN to the peripheral cytoplasm[11]. An additional population of GFP–GGA1, however, is associated with relatively immobile foci distributed throughout the cytoplasm[11]. To determine whether these peripheral

foci correspond to endosomes, HeLa cells were allowed to internalize rhodamine-conjugated transferrin (Rh–Tf) for 10 min at 37 °C and then immunostained for endogenous GGA1. This analysis revealed a high degree of co-localization between internalized Rh–Tf and endogenous GGA1 (Fig. 1a–c). Similar analyses could not be performed for endogenous GGA2 and GGA3 because the available antibodies are not sensitive enough for immunofluorescence microscopic detection of faint cytoplasmic structures. However, internalized Rh–Tf did co-localize with transiently expressed GFP–GGA1, GFP–GGA2 and GFP–GGA3 on peripheral cytoplasmic foci (Fig. 1d–l). These observations raised the possibility that the GGAs also have a role in endosomes, in addition to the TGN.

To investigate the physiological roles of the GGAs, we reduced their endogenous expression in HeLa cells using small-interfering RNAs (siRNA) and examined the localization of the cation-independent MPR (CI-MPR). Although we were able to decrease levels of the three GGAs with the corresponding siRNAs (Fig. 2a and data not shown), we only observed an effect on the distribution of CI-MPR in GGA3-depleted cells (Fig. 2c–e). In mock-treated cells, the bulk of the CI-MPR was found in the juxtanuclear area (Fig. 2c), indicative of its concentration at the TGN and late endosomes[12]. Reduction in the levels of the adaptor protein 1 (AP-1) complex, used here as a control, did not cause an obvious change in distribution of the CI-MPR (Fig. 2d). In contrast, depletion of GGA3 resulted in accumulation of the CI-MPR in cytoplasmic vesicles, some of which were very large (Fig. 2e, arrows). Many of these structures could be loaded with Rh–Tf internalized during a 10-min pulse (Fig. 2f–h), indicating that they correspond to early endosomes. Thus, depletion of GGA3 shifts the steady state localization of CI-MPR from the TGN and late endosomes to early endosomes. A possible interpretation of these observations is that the absence of GGA3 prevented the concentration of the CI-MPR in clathrin-coated areas of the TGN, resulting in mis-sorting of the receptor to the plasma membrane and subsequent cycling between the plasma membrane and endosomes.

However, additional immunofluorescence microscopy analyses hinted at a more general effect of GGA3 depletion on endosomal trafficking. We observed extensive co-localization of the CI-MPR

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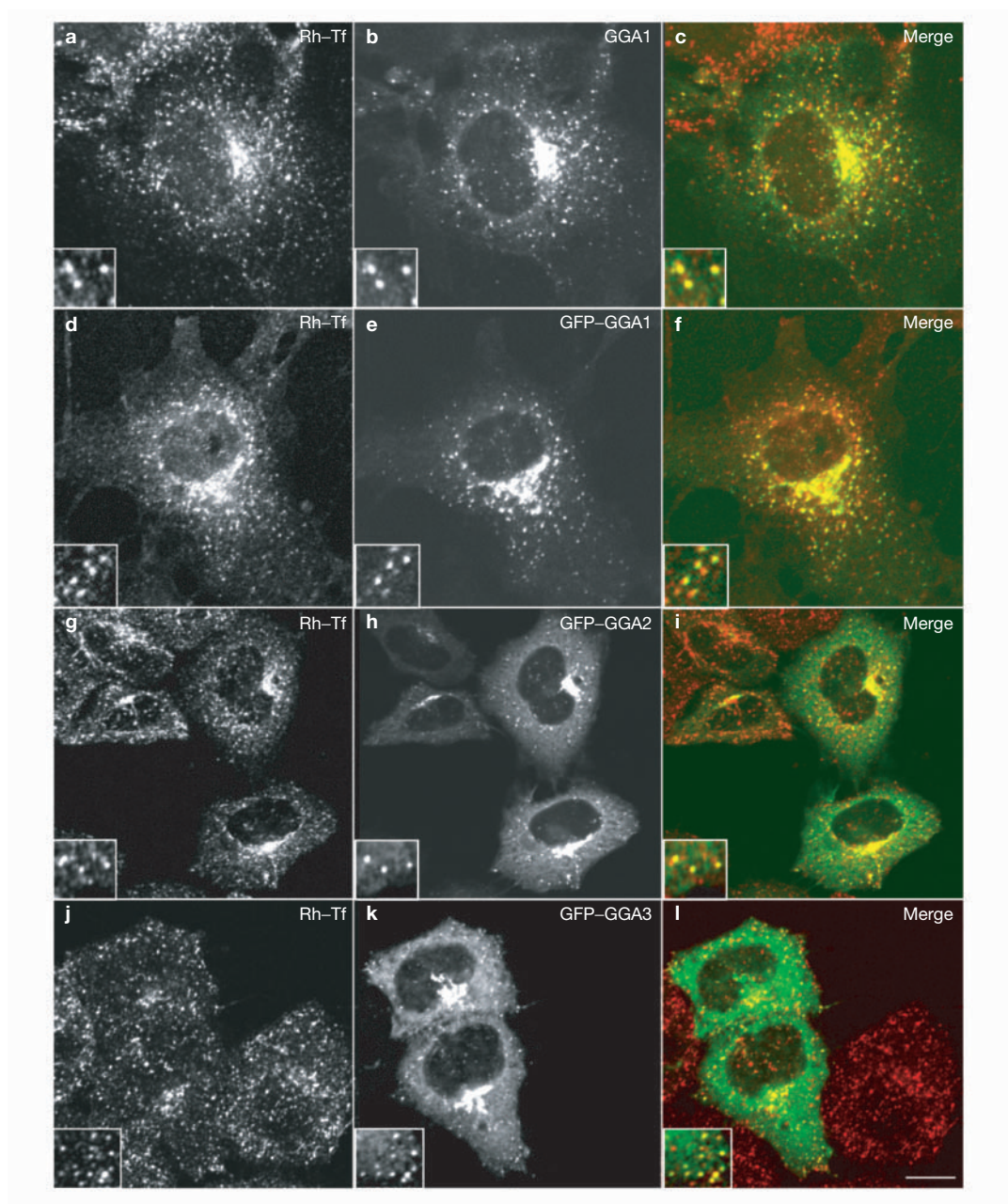[2]Correspondence should be addressed to J.S.B. (e-mail: juan@helix.nih.gov)

Figure 1 Localization of the GGAs to endosomes. HeLa cells (**a–c**) or HeLa cells transfected with GFP–GGA1 (**d–f**), GFP–GGA2 (**g–i**) or GFP–GGA3 (**j–l**), were incubated with Rh–Tf for 10 min at 37 °C to load early endosomes. Cells were fixed, permeabilized, and examined by confocal fluorescence microscopy. **a**, **d**, **g** and **j**, rhodamine fluorescence (red); **b**, immunostaining with a rabbit antibody to GGA1 followed by Cy3-conjugated donkey anti-rabbit IgG (green); **e**, **h** and **k**, GFP fluorescence (green). Insets show twofold-magnified views of peripheral cytoplasmic regions. Scale bar represents 20 μm.

with Alexa-conjugated EGF 20 min after internalization by receptor-mediated endocytosis in GGA3-deficient cells (Fig. 2i–k). At this time, most of the internalized EGF should have reached late endosomes and lysosomes[13,14], and should not have co-localized so extensively with CI-MPR that accumulated in early endosomes. This observation prompted us to analyse the localization of internalized EGF in more detail. Thus, we compared the distribution of Rh–Tf and Alexa–EGF after 30 min of simultaneous internalization in control, AP-1-depleted and GGA3-depleted cells (see Supplementary Information, Fig. S1). In control and AP-1-depleted cells, the overall appearance of EGF- and Tf-containing

endosomes was clearly distinct (see Supplementary Information, Fig. S1a–f), whereas in GGA3-depleted cells both EGF and transferrin co-localized to large endosomal structures (see Supplementary Information, Fig. S1g–i), indicating that both proteins entered a compartment from which they could no longer be sorted efficiently. Many of these structures contained early endosomal antigen (EEA1), confirming that they were early endosomal in nature (see Supplementary Information, Fig. S1j–l).

To determine the consequences of accumulating internalized EGF in enlarged early endosomes, we examined the fate of the internalized EGF over time. Control, AP-1-depleted and GGA3-depleted
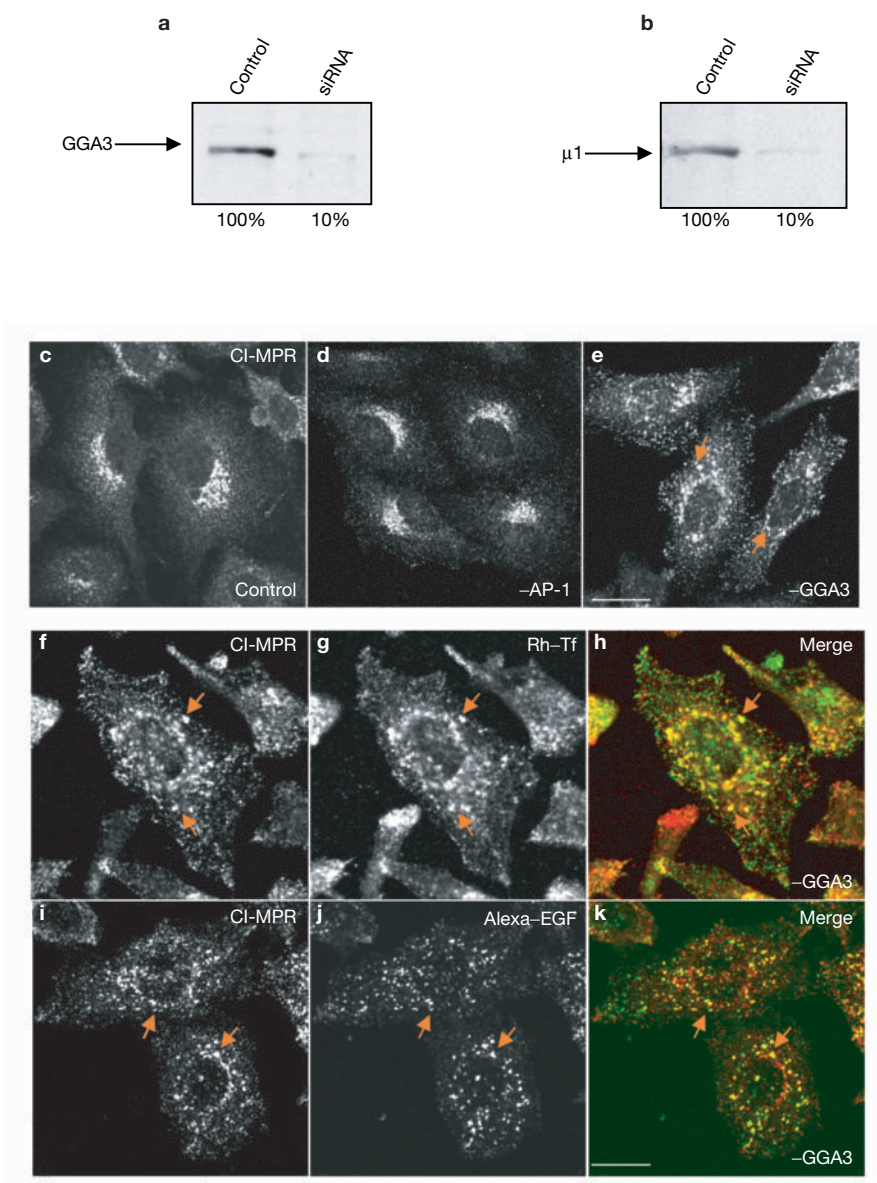
Figure 2 Depletion of GGA3 affects the localization of the CI-MPR. Equal protein loadings of homogenates from either control mock-treated cells or cells treated with siRNAs directed against GGA3 (**a**) or the AP-1 μ1 subunit (**b**) were analysed by SDS–PAGE before immunoblotting with the indicated antibody. Percentages indicate the relative amounts of the targeted protein in each sample. (**c–e**) HeLa cells were mock-treated (**c**) or treated with siRNA against μ1 (**d**) or GGA3 (**e**), immunostained for the CI-MPR and examined by confocal fluorescence microscopy. (**f–k**) HeLa cells treated with siRNA against GGA3 were allowed to internalize surface-bound Rh–Tf (**f–h**) or Alexa488–EGF (**i–k**) for 15 min at 37 °C and then immunostained for the CI-MPR. **f**, **i**, CI-MPR; **g**, rhodamine fluorescence (red); **j**, Alexa fluorescence (green). Arrows in **e–k** indicate enlarged cytoplasmic structures induced by GGA3 depletion. Scale bar represents 20 μm.

cells were incubated with Alexa–EGF for 30 min at 4 °C before a chase for 10, 30 or 180 min at 37 °C. In both control cells (Fig. 3a–c) and AP-1-depleted cells (Fig. 3d–f) disappearance of internalized EGF was essentially complete after 3 h. In GGA3-depleted cells, however, a substantial amount of internalized EGF could still be detected at this time (Fig. 3g–i). These experiments indicate that reduced levels of GGA3 impair the degradation of internalized EGF. Taken together, the experiments described thus far indicate that depletion of GGA3 causes the accumulation of the CI-MPR, internalized transferrin and internalized EGF in an aberrant early endosomal compartment.

Intriguingly, the effects of GGA3 depletion on endosomes resembled the effects of depleting cells of the mammalian proteins HRS, STAM1, STAM2 and TSG101 (refs 15–18). These proteins are components of an endosomal machinery that selects ubiquitinated cargo, such as the EGFR and other signalling receptors, for delivery to the lumen of multivesicular bodies (MVBs), a form of late endosome[14,19]. Their roles in this process rely on their ability to bind ubiquitin[19–21]. These considerations prompted us to test whether the GGAs also interacted with ubiquitin. Indeed, we observed binding of GGA3 from a HeLa cell lysate to ubiquitin–agarose, but not protein–agarose (Fig. 4a). This binding was specific, as neither
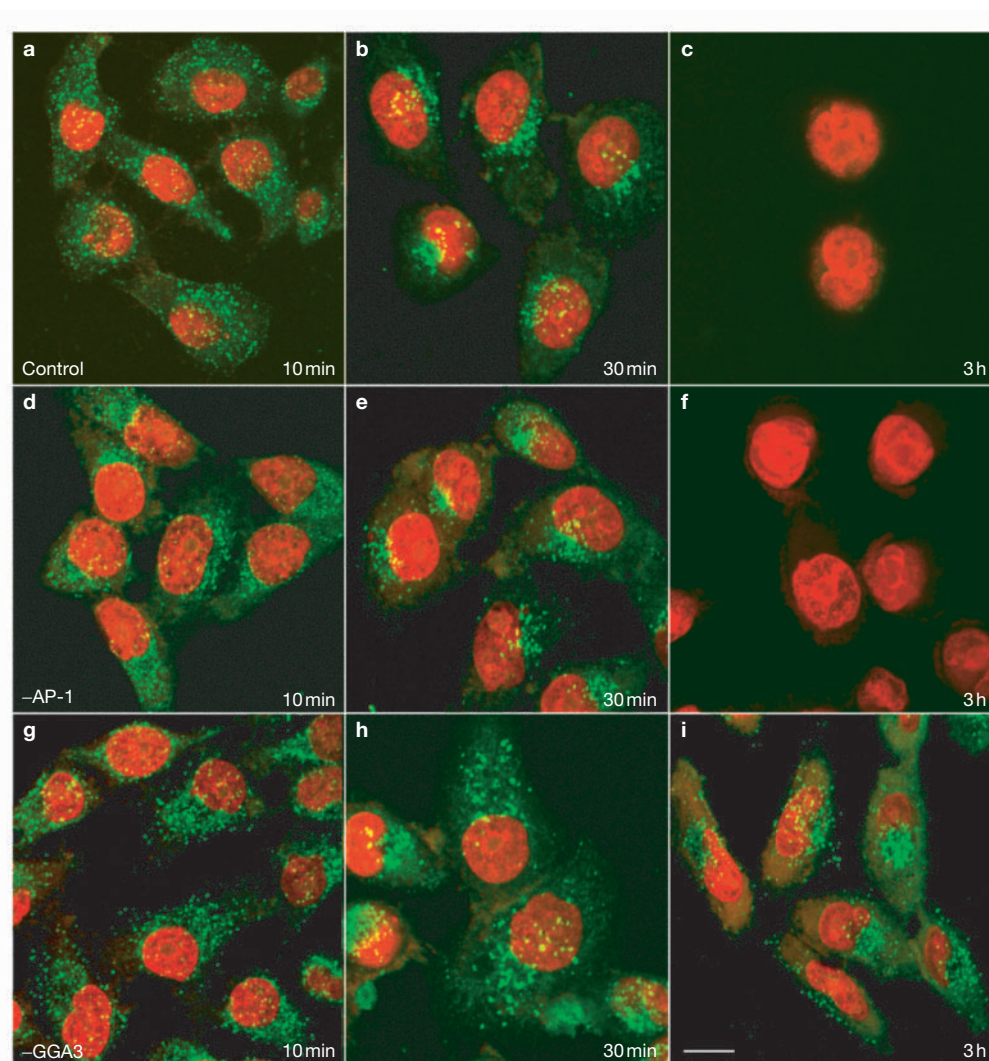
Figure 3 Depletion of GGA3 inhibits EGF degradation. The fate of surface-bound Alexa488–EGF at 37 °C was analysed in mock-treated HeLa cells (**a**–**c**) and in HeLa cells treated with specific siRNA against μ1 (**d**–**f**) or GGA3 (**g**–**i**). Cells were examined at the indicated times for Alexa488–EGF (green) and DAPI (red) staining. Confocal settings were identical for all samples. Scale bar represents 20 μm.

clathrin, AP-1 nor tubulin bound to ubiquitin (Fig. 4a). In addition, we observed that Myc-tagged versions of GGA1, GGA2 and GGA3 transiently expressed in HeLa cells also bound ubiquitin specifically, with Myc–GGA3 exhibiting the highest avidity (Fig. 4b).

To characterize further the interaction between GGA3 and ubiquitin, full-length GGA3, as well as VHS, GAT, VHS–GAT, hinge and GAE fragments of GGA3, were tested for interactions using the yeast two-hybrid system. This methodology confirmed the interaction of GGA3 with ubiquitin (Fig. 4c). A construct comprising both the VHS and GAT domains bound ubiquitin well, but the isolated VHS, GAT, hinge or GAE domains showed little or no binding (Fig. 4c). Substitution of Ala for Asn 91, a mutation that impairs the ability of the VHS domain to interact with acidic-cluster-dileucine signals[22], did not affect the interaction of VHS–GAT with ubiquitin (Fig. 4d). In addition, a VHS–GAT domain derived from a naturally occurring splice variant of GGA3 that lacks residues 68–101 and is unable to interact with acidic-cluster-dileucine signals[34], could still bind ubiquitin (Fig. 4d). Replacement of Ala for Asn 194, which is involved in

binding of the GAT domain to Arf[7], similarly failed to prevent the interaction with ubiquitin (Fig. 1d). These observations indicate that the interaction of the VHS–GAT region of GGA3 with ubiquitin involves residues that are distinct from those previously implicated in the binding to acidic-cluster-dileucine signals or Arf. However, substitution of Ala for Leu 276, which is part of a hydrophobic patch on the carboxy-terminal portion of the GAT domain[23,24], completely abrogated binding to ubiquitin (Fig. 4d), even though it did not affect binding to Arf or acidic-cluster-dileucine signals (data not shown). Therefore, this hydrophobic patch in the GAT domain is involved in VHS–GAT–ubiquitin interactions. Mutational analyses of ubiquitin demonstrated that Ile 44 is required for the interaction with GGA3 (Fig. 4e). This residue is part of a hydrophobic patch on ubiquitin that mediates binding to the proteasome, as well as to the CUE, UIM and UBA domains[20,25–28]. In contrast, mutations in Leu 8 and Ile 36, two residues implicated in the interaction with other ubiquitin-binding domains[28,29], or in Phe 4, which is important for the ubiquitin-dependent internalization of α-factor[27], did not affect

a

b

c

d
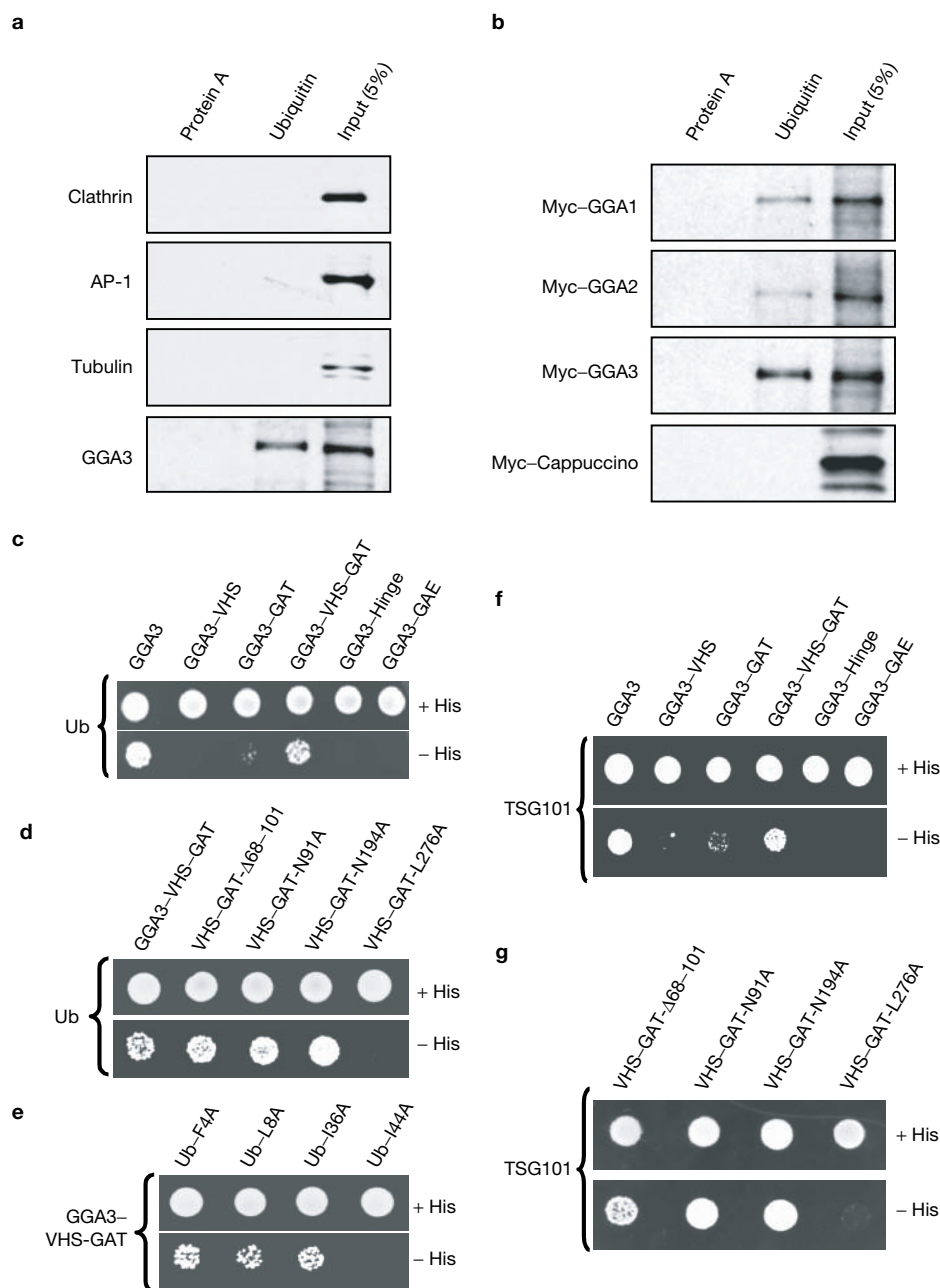

e

f

g

Figure 4 Interaction of GGAs with ubiquitin and TSG101. (**a**, **b**) Pull-down assays for ubiquitin (Ub) binding. Lysates from HeLa cells (**a**), or HeLa cells transiently transfected with Myc–GGA1, Myc–GGA2, Myc–GGA3 or Myc–Cappuccino (**b**), were incubated with ubiquitin–agarose or protein-A–agarose, and bound proteins were detected by immunoblotting for the indicated proteins. (**c**–**g**) Two-hybrid assays. Yeast transformants expressing the combinations of constructs indicated in the figure were spotted onto plates lacking leucine and tryptophan, with or without histidine (+ His and – His, respectively).

the interaction between GGA3 and ubiquitin. These results are consistent with other observations published while our manuscript was under revision[30].

The ability of HRS to target ubiquitinated cargo to the MVB pathway depends on its ability to interact with TSG101 (refs 15, 18, 31). Yeast two-hybrid experiments showed that full-length GGA3 also interacted with TSG101 and that this interaction was mediated by the VHS–GAT domains (Fig. 4f). Moreover, mutational analyses

revealed that binding to TSG101 also required Leu 276 of GGA3 (Fig. 4g). siRNA-mediated depletion of TSG101 did not affect the ability of ubiquitin to bind GGA3 (see Supplementary Information, Fig. S2), indicating that ubiquitin–GGA3 interactions occur independently of TSG101.

The accumulation of internalized Alexa–EGF in the enlarged early endosomes of GGA3-depleted cells could be reversed by expression of siRNA-resistant, long (Fig. 5a–c) and short (Δ68–101; Fig. 5d–f)
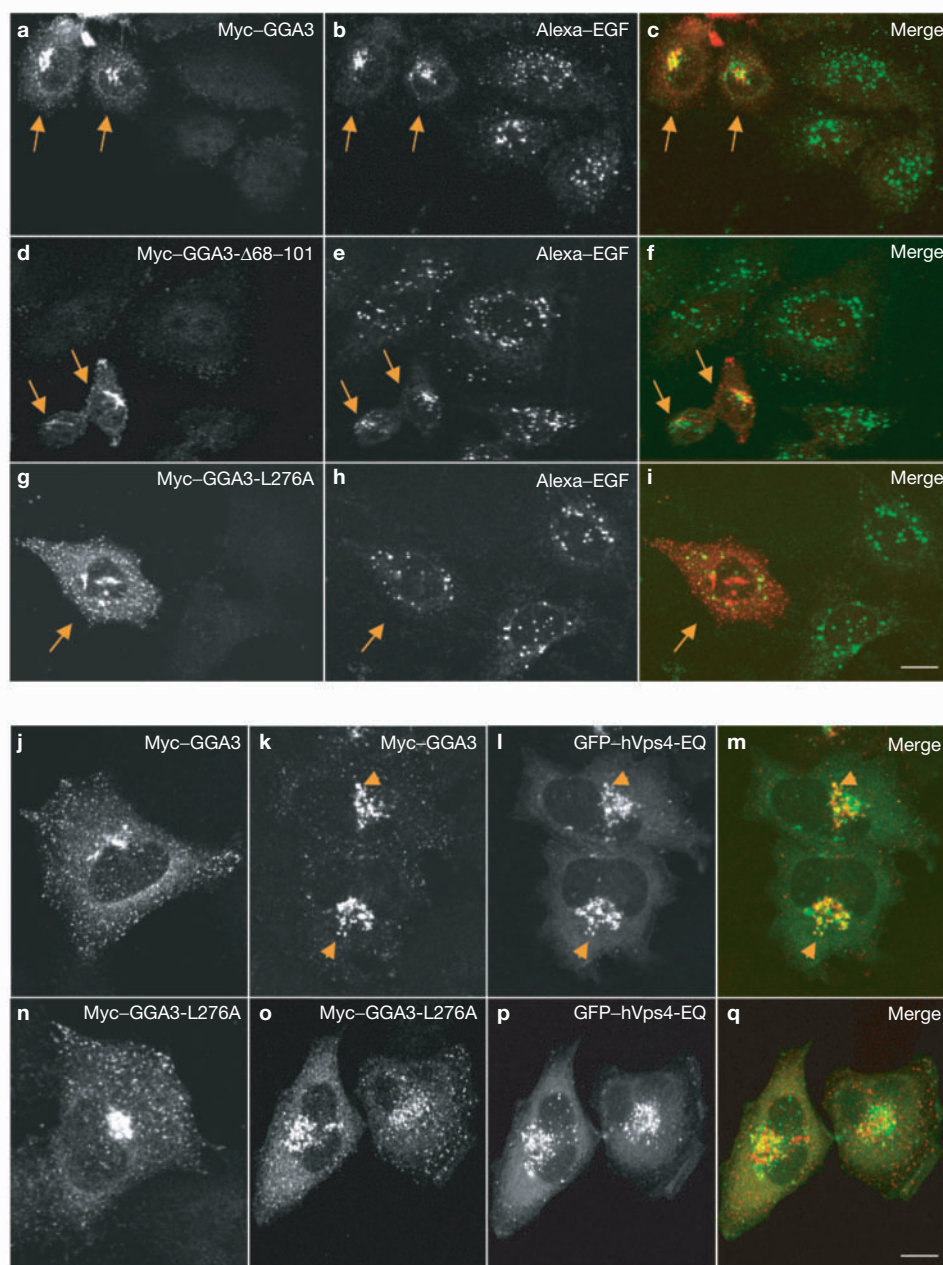
Figure 5 Interaction of GGA3 with ubiquitin is required for endosomal sorting. (a–i) HeLa cells treated with GGA3 siRNA were transfected with RNAi-resistant versions of Myc–GGA3 (a–c), Myc–GGA3-Δ68–101 (d–f) or Myc–GGA3-L276A (g–i). At 15 h after transfection, cells were allowed to internalize surface-bound Alexa488–EGF for 20 min at 37 °C, fixed, permeabilized and stained with a mouse antibody to the Myc epitope followed by Cy3-conjugated donkey anti-mouse IgG. a, d and g, Myc staining (red); b, e and h, Alexa488 fluorescence (green). Scale bar represents 10 μm. Arrows indicate transfected cells. (j–q) Hela cells transfected with Myc–GGA3 and Myc–GGA3-L276A alone (j, n) or cotransfected with a GFP-tagged, ATPase-deficient, form of human Vps4 (GFP–hvps4-EQ)[32] (k–m and o–q) were fixed, permeabilized, stained with a mouse antibody to Myc followed by Cy3-conjugated donkey anti-mouse IgG, and observed by confocal fluorescence microscopy. j, k, n and o, Myc staining (red); l, p, GFP fluorescence (green). Scale bar represents 5 μm. Arrowheads indicate co-stained vesicles.

isoforms of Myc–GGA3, but not by expression of the Myc–GGA3-L276A mutant, which is unable to bind ubiquitin and TSG101 (Fig. 5g–i). In addition, expression of a dominant-negative, ATPase-deficient, mutant of human Vps4 (GFP–hVps4-EQ), which stabilizes association of TSG101 and other components of the ubiquitin sorting machinery with aberrantly enlarged MVB precursor endosomes[32] (that is, the 'class E' compartment[19]), resulted in accumulation of Myc–GGA3 (Fig. 5j–m) but not Myc–GGA3-L276A (Fig. 5n–q) on those endosomes. Therefore, the ability of GGA3 to interact with ubiquitin and TSG101 is required for endosomal sorting of internalized EGF and for association of GGA3 with the class E compartment.

In conclusion, we have demonstrated the existence in HeLa cells of a population of GGAs on endosomes, and shown that depletion

of GGA3 results in the accumulation of CI-MPR and internalized EGF in enlarged early endosomes. These effects are not likely to be a consequence of mis-sorting of transmembrane proteins bearing acidic-cluster-dileucine signals, as the most abundant species of GGA3 in HeLa cells is the Δ68–101 variant[33], which binds ubiquitin (this study) but not acidic-cluster-dileucine signals[34]. Moreover, re-expression of the Δ68–101 variant in GGA3-depleted cells is sufficient to restore the endosomal sorting of internalized EGF. Thus, the effects of GGA3 depletion reported here most probably reflect a function of this protein in endosomes, which may be mediated by its interactions with the ubiquitin sorting machinery. GGA3 might therefore function as an endosomal ubiquitin adaptor in a manner analogous to HRS. Nonetheless, these considerations do not exclude the possibility that the GGAs could also function in ubiquitin-mediated sorting at the TGN and that some of the effects reported here could be an indirect result of mis-sorting of ubiquitinated proteins from the TGN. Finally, our results indicate that despite their similarities, the three human GGAs are not entirely redundant. GGA3, in particular, is either more important or more abundant than the other two in HeLa cells, such that the absence of GGA3 alone results in defective morphology and function of early endosomes. □

METHODS

siRNA. Two siRNAs against human GGA3 (5′-AAAAACGGCUUCCG-CAUCCUC-3′), and μ1 (5′-AAGGCAUCAAGUAUCGGAAGA-3′) were designed and synthesized as Option C by Dharmacon Research (Lafayette, CO). Two consecutive transfections every 72 h were carried out in HeLa cells according to the manufacturer's instructions. For rescue assays, cytidines at positions 1818, 1824, and 1830 of Myc–GGA3, Myc–GGA3-Δ68–101 and Myc–GGA3-L276A were mutated to two guanines and one adenine, respectively, to generate RNAi-resistant versions of the proteins without changing the encoded amino acids.

Internalization assays. Control cells or cells treated with specific siRNAs were placed on ice and washed with pre-chilled serum-free medium (DMEM containing 20 mM Hepes) containing 1% BSA (SFM). Cells were then incubated for 30 min at 4 °C in SFM containing either Alexa488–EGF (5 μg ml^{-1}), rhodamine–transferrin (10 μg ml^{-1}), or both (Molecular Probes, Eugene, OR), then washed with PBS and incubated at 37 °C for various times. Cells were examined using an inverted confocal laser-scanning microscope (LSM 510; Carl Zeiss, Thornwood, NY).

Pull-down assays. Control cells or cells expressing Myc-tagged versions of GGA1, GGA2, GGA3 or Cappuccino, were lysed in 25 mM Tris-HCl at pH 7.4, 150 mM sodium chloride, 5 mM EDTA and 0.5% Triton X-100 (TNET), and incubated with ubiquitin–agarose or protein-A–agarose (Sigma, St Louis, MO) for 2 h at 4 °C. After washing with TNET, samples were boiled in Laemmli sample buffer and resolved by SDS–PAGE before immunoblotting.

Other procedures and reagents. Wild-type ubiquitin and ubiquitin mutants were obtained by RT-PCR amplification with specific primers and then subcloned into the pGBT9 (TRP1) vector (Clontech, Palo Alto, CA). Plasmids encoding the following proteins were gifts of the investigators indicated in parenthesis: TSG101 (S. Cohen; Stanford University, CA, USA), GFP–hVps4–EQ (P. Woodman, University of Manchester, UK) and the short isoform of GGA3 (Δ68–101; R. Kahn, Emory University School of Medicine, GA). Cloning of the different domains of GGA3, as well as Myc-epitope and GFP-tagged versions of the GGAs, was described previously[2,4,11].

Cell culture and transfection, immunofluorescence and yeast two-hybrid assays were performed as previously described[2]. The following antibodies were used in this study: mouse monoclonal antibody to γ1-adaptin (100/3) and tubulin (Sigma); mouse monoclonal antibody to the CI-MPR (Research Diagnostics Inc., Flanders, NJ); mouse monoclonal antibody to TSG101 (Santa Cruz Biotechnology, Santa Cruz, CA); mouse monoclonal (9E10) and rabbit polyclonal antibodies to the Myc epitope (Covance, Princeton, NJ);

mouse monoclonal antibody to clathrin (CHC), EEA1 and GGA3 (Transduction Laboratories, Lexington, KY). A polyclonal antibody to GGA1 was a gift of M. S. Robinson (University of Cambridge, UK).

Note: Supplementary Information is available on the Nature Cell Biology website.

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